Research article

Longevity of sperm cells retrieved by post-mortem epididymal aspiration in wild bovids in zoo conditions

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Abstract

The conservation of endangered wildlife species may depend on the development of assisted reproductive technologies, and work in this field has focused on epididymal sperm conservation. Epididymes from wild Bovidae (24 individuals from 13 species) kept in captivity at the Réserve Africaine de Sigean (France) were collected quickly after death and stored at +4° C. Sperm motility, viability and morphology were regularly examined at various time intervals. Sperm motility and viability were significantly lower after several days ($P < 0.05$). There was no significant difference in the decrease in sperm motility and viability between sub-families ($P > 0.05$). The most represented morphology was sperm cells with a cytoplasmic droplet. Head and flagellum abnormalities and sperm cells without a flagellum increased during low-temperature storage, significantly after several days. Epididymal sperm quality appeared independent of seasonality. This work is the first report of this technique in zoo conditions with limited equipment, and the data obtained matches previously published results. About 30% of epididymal sperm survived for three days at +4° C and almost 10% survived for five days, and it should be possible to use these sperm cells in some assisted reproductive technologies several days after the animal's death.

Introduction

Bovids represent a diverse family of almost 140 species divided into 10 sub-families, the main ones being Bovinae, Caprinae and Antilopinae. These wild ruminants are frequently represented in zoo collections worldwide. However, knowledge of these species – which are fearful, potentially dangerous to handle and notoriously difficult to anaesthetise – remains quite limited, particularly with regard to reproduction. Thus, the development of assisted reproductive technology in wild bovids is still in its infancy compared to other families.

On the other hand, zoo breeding programmes have existed for 30 years in Europe and North America to optimise the diversity of these populations. Many animals are exchanged among participating institutions; however, the transport of wild Bovidae remains difficult and many deaths are recorded each year, which is counter-productive and reduces the genetic diversity of the captive population.

To maintain biodiversity and minimise the negative consequences of its loss on small or fragmented populations, the development of techniques for preserving the gametes of endangered species is essential. With the progress already made on assisted reproductive technologies – artificial insemination

and cryopreservation – and the growing awareness of the need to protect many endangered species, genome banks have been established in recent years (Andrabi et al. 2007; Leibo et al. 2002; Pukazhenthi et al. 2004).

Epididymal sperm collection is relatively well documented in wild Cervidae (Martinez et al. 2008; Martinez-Pastor et al. 2005; Soler et al. 2003), but few data are available on wild Bovidae (Bezuidenhout et al. 1995; Bissett et al. 2005; Chatiza et al. 2011; Saragusty et al. 2006), and most concern Cape buffaloes (*Syncerus caffer*) (Bartels et al. 1999, 2001; Friedmann et al. 2000; Kilian et al. 2000). The advantage of this technique is undoubtedly post-mortem sperm collection, especially as numerous deaths are recorded with no prodrome in wild Bovidae in zoo collections. Spermatozoa stored in the cauda epididymis are fertile and have already undergone almost all their maturation, making it a good source of gametes (Bissett et al. 2005; Chatiza et al. 2011; Santiago-Moreno et al. 2006). Several techniques have previously been described for the extraction and recovery of epididymal sperm: section of the cauda epididymis, pressure, or flushing with a suitable medium through the vas deferens. Flushing proved to be a superior technique due to lower risk of contamination; however, this technique seems less suited to work in the field (Martinez-

Pastor et al. 2006) as it requires more material and extender. Moreover, if the gametes obtained are stored, this provides an opportunity to extend a male's reproductive career into the future: with appropriate preservation conditions for each species, the spermatozoa collected can be used for future inseminations (Bartels et al. 2001; Guard et al. 1995).

The aims of our study were to investigate the effects of low temperature storage on epididymal spermatozoa lifetime and quality, following the death of donors. We conducted our study on captive exotic ruminants using equipment available in classic zoological practice, and then compare our results with previous in situ work.

Methods

All manipulations (necropsies, sperm collection and analysis) were performed by a single technician in order to eliminate interindividual variability. The manipulations were also done with limited equipment: no computer-assisted techniques or extender were used.

Epididymis collection and sperm extraction and preparation

Epididymes were recovered post-mortem from 24 sexually mature Bovidae males belonging to 13 species that died at the Reserve Africaine de Sigean (France) throughout one year. The animals included in this study belong to the following subfamilies and species (listed in brackets is the number of individuals for each species): Bovinae – sitatunga (*Tragelaphus spekei*) (n = 2), greater kudu (*Tragelaphus strepsiceros*) (n = 1), Cape eland (*Taurotragus oryx*) (n = 1), dwarf forest buffalo (*Syncerus caffer nanus*) (n = 1); Antilopinae – Southern lechwe (*Kobus leche*) (n = 4), Defassa waterbuck (*Kobus ellipsiprymnus defassa*) (n = 1), springbok (*Antidorcas marsupialis*) (n = 5), impala (*Aepyceros melampus*) (n = 1), blue wildebeest (*Connochaetes taurinus*) (n = 2), blesbok (*Damaliscus pygargus phillipsi*) (n = 1), gemsbok (*Oryx gazella*) (n = 3); Caprinae – Barbary sheep (*Ammotragus lervia*) (n = 1), mufflon (*Ovis ammon musimon*) (n = 1).

All of these animals lived in environmental and social conditions that followed the welfare and captive breeding recommendations for the species concerned. All the donors were living in mixed-sex herds, housed in open enclosures with access to shelters, and fed a combination of pellets and hay.

Each individual received a complete necropsy to establish the cause of death. Any death due to infectious, debilitating, chronic inflammatory or nutritional disease was excluded from the study. Similarly, animals given any pharmacological treatment with a deleterious effect on male reproductive function within two months prior to their death were not included in the study. Necropsies were conducted in accordance with practice and health requirements.

For 16/24 (66.7%) individuals, death was noted without prodrome, so the exact time of death is unknown, and was arbitrarily set at 0000 on the day of death.

The testes of each subject included in the study were taken from each cadaver and placed in a cold room at +4° C as soon as possible after confirmation of death. A macroscopic examination of the testes, epididymes and their tunica was undertaken in order to detect any abnormality or disease that would disqualify the individual from the study. To avoid excessive drying, the testes were stored in their tunica for the remainder of the study.

Epididymal sperm collection was performed using a 25-gauge needle mounted on a 1 ml syringe previously filled with 0.5 ml of a sterile isotonic liquid preparation (Lactated Ringer's solution, Aguettant, Lyon, France). Ringer's lactate was selected as diluant as it was easy to obtain and no extender was available. Moreover, its isotonicity is closer to that of epididymal fluid than is sodium

chloride's. The procedure was performed by catheterising the epididymal duct in the cauda epididymis and aspirating some epididymal fluid in the syringe. Collection by puncture directly into the epididymal duct can avoid blood contamination as described by Martinez-Pastor et al. (2006) during the sectioning of the *cauda epididymis*. Sperm collection was performed at room temperature, and done as quickly as possible to limit temperature fluctuation. The epididymes were then placed at 4° C for subsequent collections: aspirations were repeated randomly during the following few days until the detection of a significant immobility of the spermatozoa (121 to 190.5 hours after the estimated time of death). Most studies are done by removing the epididymal sperm shortly after the donor's death, and doing all subsequent studies in an extender (Martinez et al. 2008; Martinez-Pastor et al. 2005; Soler et al. 2003). We chose to keep the sperm in the epididymis as we did not use extender, and so to limit dessication of epididymal sperm. The purpose of our study was to determine if viable gametes could be retrieved from a corpse several days after death; keeping the sperm in the epididymis was thus closer to the desired conditions, and so exposed sperm cells to tissue degradation by-products.

Sperm quality analysis

101 microscopic analyses were performed on the 24 animals studied, an average of 4.2 examinations per individual, with a range of two to seven.

To assess the quality of the sperm collected, the following parameters were chosen: individual motility, morphology and vitality.

Individual motility

The epididymal sperm collected by needle aspiration was gradually warmed to 37° C. Individual motility was assessed subjectively by light microscopy at x100 magnification on at least five fields, with a minimum of 200 spermatozoa per examination. Microscopic analysis of individual motility was made by depositing a drop of liquid obtained on a microscope slide previously maintained at 37° C, soon after collection. The percentage of motile sperm was then recorded.

A subjective rate was given to each spermatozoon using a scale of 0–5 (0: no movement, to 5: linear and rapid motion) (Baril et al. 1993; Blash et al. 2000; Soler et al. 2003).

To better represent the proportion of sperm from each of these categories among the total motile sperm, a Sperm Motility Index (SMI) was calculated using the following formula: SMI = Σ[percentage of motile spermatozoa + (20 x motile sperm rate)]/2 (Martinez-Pastor et al. 2005; Saragusty et al. 2006; Soler et al. 2003).

Morphology

A drop of the sperm suspension obtained was placed between a slide and coverslip and observed under light microscopy at x400 magnification, on at least five fields, with a minimum of 200 sperm per examination. The sperm was not stained for this analysis. The morphological differences found were grouped as follows: normal spermatozoa, spermatozoa with a head abnormality, with an abnormal flagellum, flagellumless spermatozoa, sperm with a cytoplasmic droplet (proximal or distal) (Baril et al. 1993). The results were then compiled for each sample to calculate the proportions of each morphology.

Vitality

Epididymal sperm vitality was determined by assessing the percentage of live and dead gametes using eosin-nigrosin stain (Baril et al. 1993; Blash et al. 2000; Saragusty et al. 2006) with a rapid sperm analysis staining kit (kit Vita-eosin, Réactifs RAL,
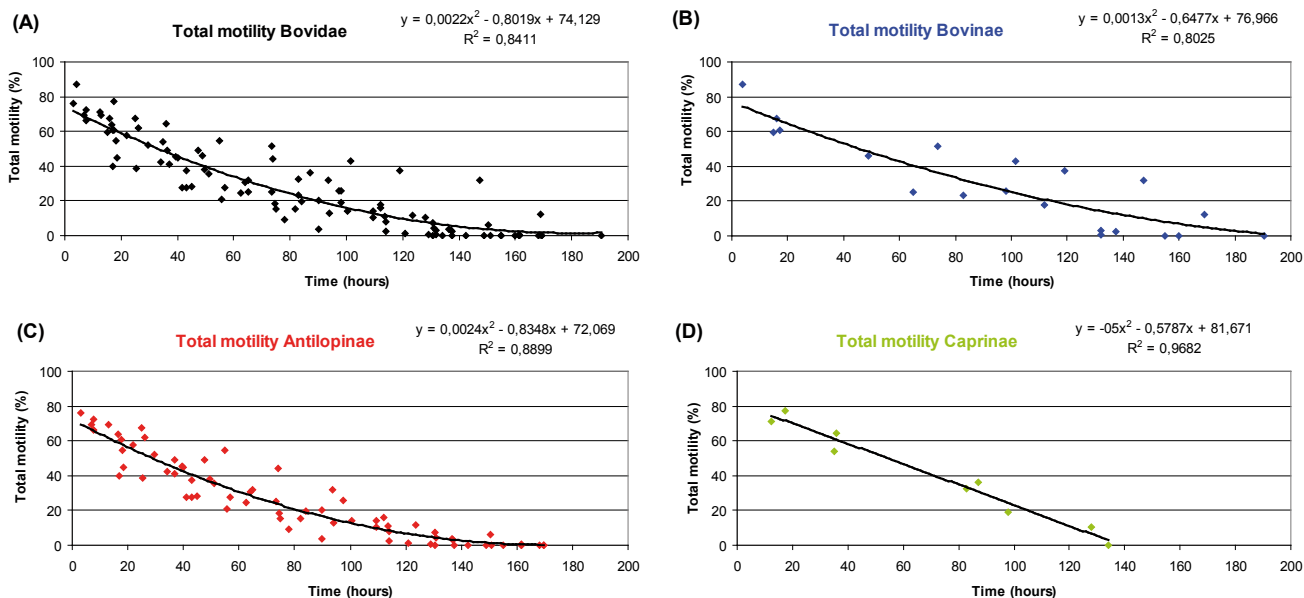
Figure 1. Epididymal sperm total motility over time. (A) Family Bovidae. (B) Subfamily Bovinae. (C) Subfamily Antilopinae. (D) Subfamily Caprinae.

Martillac, France). Microscopic observation was performed at x400 magnification of the sample prepared following the manufacturer's recommendations; the dead spermatozoa appear coloured in pink, the living colourless, and the background of the smear is stained dark blue.

Statistical analysis

Data analysis was undertaken with the statistical analysis software SPAD 5 (Coheris-Spad, Suresnes, France) and spreadsheet software and graphic design Excel 2000 (Microsoft Office, Microsoft Corporation, Redmond, Washington, USA). Data are presented as follows: mean ± 1 standard deviation. An ANOVA was used

to test for significant differences in parameters over time. The comparison of means and equations of regression curves obtained necessitated the use of Fischer–Student's and Fischer–Snedecor's tests in respect of compliance testing. The statistical error for the different tests was set at $p < 0.05$.

Results

Motility

The storage of epididymes collected post-mortem at low temperature (+4° C) induced a statistically significant decrease in epididymal sperm motility over time. The calculation of the
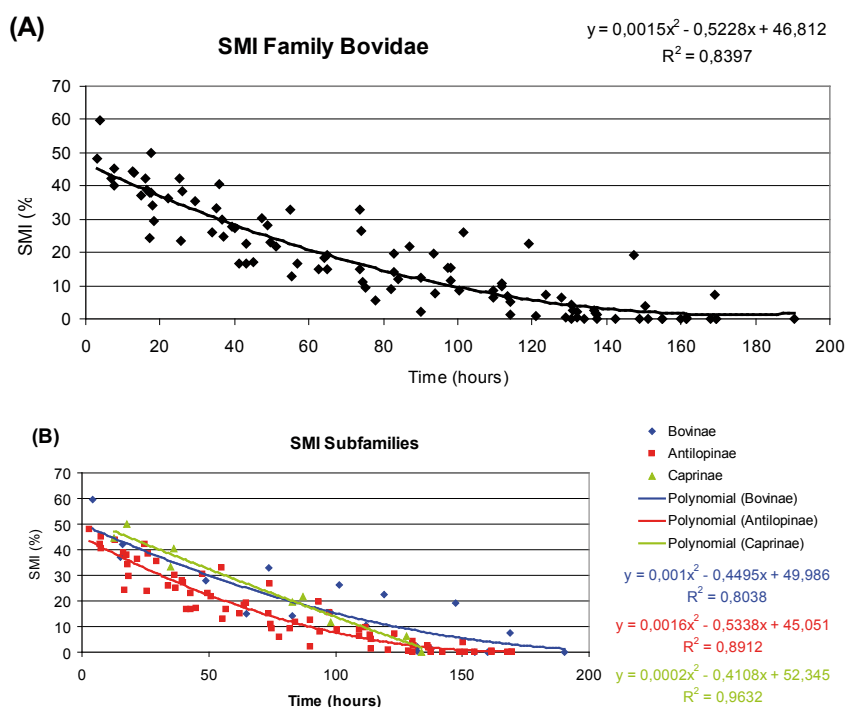

Figure 2. Sperm Motility Index (SMI) over time. (A) Family Bovidae. (B) Subfamilies Bovinae, Antilopinae and Caprinae.

Table 1. Changes in epididymal sperm morphology over time. Results are expressed as percentages (%), and presented as mean ± standard deviation. Different letters on the same line represent statistically significant differences ($p < 0.05$) in comparison to the first time period (0–24h). The number of samples is given in parentheses.

Parameters	Time (hours)							
	0–24 (n=17)	24–48 (n=16)	48–72 (n=10)	72–96 (n=15)	96–120 (n=13)	120–144 (n=15)	144–168 (n=10)	> 168 (n=5)
BOVIDAE (n = 101)								
Normal	37.76 ± 5.51	38.13 ± 5.47[a]	41.03 ± 4.32[a]	38.74 ± 4.51[a]	39.61 ± 4.05[a]	37.55 ± 3.15[a]	37.15 ± 3.62[a]	38.76 ± 1.29[a]
Head abnormalities	6.18 ± 2.42	6.31 ± 1.94[a]	7.10 ± 1.53[a]	6.90 ± 2.08[a]	6.67 ± 1.87[a]	7.34 ± 2.02[a]	6.81 ± 2.02[a]	7.82 ± 1.11[b]
Flagellum abnormalities	7.21 ± 3.11	8.30 ± 3.87[a]	7.21 ± 2.99[a]	8.62 ± 3.44[a]	7.97 ± 3.09[a]	10.09 ± 3.44[a]	10.25 ± 3.70[a]	9.17 ± 1.67[b]
No flagellum	1.22 ± 0.67	1.37 ± 0.74[a]	1.44 ± 0.96[a]	1.78 ± 0.83[a]	1.82 ± 0.65[a]	1.57 ± 0.79[a]	1.93 ± 0.83[a]	2.27 ± 1.13[b]
Cytoplasmic droplet	47.56 ± 4.63	46.20 ± 4.54[a]	44.09 ± 3.56[a]	44.04 ± 3.24[a]	44.09 ± 2.79[a]	43.45 ± 2.85[a]	43.66 ± 2.45[a]	41.98 ± 3.32[b]
Bovinae (n = 20)								
Normal	37.47 ± 4.81	–	41.96 ± 0.36[a]	40.05 ± 6.17[a]	36.42 ± 4.10[a]	35.42 ± 4.81[a]	38.40 ± 4.13[a]	38.21 ± 2.21[a]
Head abnormalities	7.14 ± 3.54	–	5.80 ± 1.08[a]	5.34 ± 1.08[a]	8.37 ± 2.24[a]	7.93 ± 3.42[a]	7.01 ± 1.05[a]	7.44 ± 0.81[a]
Flagellum abnormalities	6.02 ± 2.67	–	3.52 ± 1.15[a]	7.07 ± 3.04[a]	7.67 ± 3.02[a]	10.00 ± 4.64[a]	8.57 ± 3.83[a]	7.99 ± 1.11[b]
No flagellum	1.03 ± 0.28	–	1.40 ± 0.74[a]	1.21 ± 0.18[a]	2.18 ± 0.72[a]	1.99 ± 1.24[a]	1.48 ± 0.69[b]	1.85 ± 0.26[b]
Cytoplasmic droplet	48.07 ± 1.73	–	47.31 ± 0.32[a]	46.31 ± 1.87[a]	45.36 ± 1.84[a]	44.66 ± 3.14[b]	44.55 ± 3.34[b]	44.49 ± 2.77[b]
Antilopinae (n = 72)								
Normal	37.87 ± 6.43	38.26 ± 5.86[a]	40.80 ± 4.87[a]	38.59 ± 4.92[a]	41.67 ± 2.86[a]	38.34 ± 2.81[a]	36.62 ± 3.59[a]	39.12 ± 0.61[a]
Head abnormalities	5.81 ± 2.26	6.29 ± 2.01[a]	7.43 ± 1.50[a]	7.26 ± 2.29[a]	5.66 ± 0.89[a]	7.30 ± 1.83[a]	6.73 ± 2.40[a]	8.08 ± 1.37[a]
Flagellum abnormalities	7.55 ± 3.52	8.41 ± 4.14[a]	8.13 ± 2.54[a]	8.96 ± 3.84[a]	7.86 ± 3.44[a]	10.39 ± 3.59[a]	10.97 ± 3.69[a]	9.95 ± 1.62[a]
No flagellum	1.26 ± 0.81	1.43 ± 0.78[a]	1.45 ± 1.05[a]	1.89 ± 0.93[a]	1.66 ± 0.62[a]	1.40 ± 0.71[a]	2.12 ± 0.85[a]	2.55 ± 1.49[b]
Cytoplasmic droplet	47.51 ± 5.70	45.98 ± 4.77[a]	43.29 ± 3.53[a]	43.41 ± 3.46[a]	43.40 ± 3.23[a]	42.56 ± 2.56[a]	43.27 ± 2.17[a]	40.30 ± 2.78[b]
Caprinae (n = 9)								
Normal	37.77 ± 1.59	37.28 ± 0.00[a]	–	38.25 ± 0.50[a]	35.82[b]	36.77 ± 0.78[a]	–	–
Head abnormalities	6.30 ± 0.04	6.43 ± 1.94[a]	–	6.48 ± 0.57[a]	8.01[b]	6.66 ± 1.17[a]	–	–
Flagellum abnormalities	7.75 ± 1.40	7.57 ± 0.90[a]	–	8.29 ± 1.53[a]	10.02[b]	8.67 ± 1.40[a]	–	–
No flagellum	1.35 ± 0.42	0.98 ± 0.13[a]	–	1.74 ± 0.06[a]	1.67[a]	1.83 ± 0.40[a]	–	–
Cytoplasmic droplet	46.82 ± 2.52	47.73 ± 2.71[a]	–	45.22 ± 2.55[b]	44.48[b]	46.07 ± 2.96[a]	–	–

y-intercept (at t = 0h, estimated time of death) revealed a total motility of 74.1%. The decrease was statistically significant from the third day, and there was a marked decrease after 120 hours (five days). To determine the characteristics of the decrease in motility of epididymal sperm over time, a trend line, regression curve type, was obtained from the data, with a high coefficient of determination ($R^2 = 0.841$) (Figure 1A). Thus, it appears that the approximation of this decline follows, at the level of the Bovidae family, a polynomial decay.

The decrease in total motility of epididymal sperm over time was studied for each subfamily (Figure 1B, C and D). The rates of motile epididymal sperm estimated at death were respectively 77.0%, 72.1% and 81.7% for the Bovinae, Caprinae and Antilopinae subfamilies. Similarly, the approximation of this decline followed, as at the level of the family, a polynomial decay with high coefficients of determination ($R^2 = 0.802$, 0.890 and 0.968 respectively for the Bovinae, Caprinae and Antilopinae).

The comparison of the decrease in epididymal sperm total motility over time, through statistical comparison of the trend line equations, showed no statistically significant difference between the subfamilies tested. However, comparison of the intercepts (reflecting total motility estimated at the time of death) showed that the percentage of motile sperm was statistically higher in Caprinae compared to other subfamilies, and to results obtained for the Bovidae family. However, the data for this subfamily were few (two individuals, nine samples), and so no real conclusions can be drawn.

The storage of epididymes collected post-mortem at 4° C also produced a statistically significant decrease in the value of the sperm motility index (SMI) over time. The SMI estimated at death was 46.8%. As previously observed for total motility, a statistically significant difference was observed from the third day, as a significant decrease after 120 hours (five days).

As before, a trend line with a high coefficient of determination ($R^2 = 0.840$) was obtained (Figure 2A); this curve followed a second order polynomial decay.

Decay over time of the sperm motility index from sperm obtained by post-mortem epididymal aspiration for the different subfamilies studied was approximated by a polynomial decay with high coefficients of determination ($R^2 = 0.804$, 0.891 and 0.963

Table 2. Morphology of epididymal sperm according to species and subfamilies. Results are expressed as percentages (%), and presented as mean ± standard deviation. Different letters in the same column represent statistically significant differences ($p < 0.05$) in comparison to the family value. The number of samples is given in parentheses.

	Morphology				
	Normal	Head abnormalities	Flagellum abnormalities	No flagellum	Cytoplasmic droplet
BOVIDAE	38.49 ± 4.44	6.78 ± 1.98	8.51 ± 3.41	1.60 ± 0.81	44.76 ± 3.85
Bovinae (n=5)	37.87 ± 4.07	7.20 ± 2.28	7.38 ± 3.21	1.61 ± 0.73	45.88 ± 2.41
Dwarf forest buffalo (*n=1*)	36.69 ± 1.59[a]	7.22 ± 2.14[a]	8.35 ± 2.97[a]	1.76 ± 0.75[a]	45.41 ± 2.61[a]
Cape eland (*n=1*)	41.40 ± 1.56[a]	7.20 ± 1.17[a]	3.73 ± 1.57[b]	2.12 ± 0.65[a]	45.30 ± 2.81[a]
Greater kudu (*n=1*)	33.56 ± 2.09[b]	7.74 ± 0.64[a]	13.64 ± 0.94[c]	0.94 ± 0.09[b]	44.10 ± 1.87[a]
Sitatunga (*n=2*)	38.28 ± 5.62[a]	7.76 ± 3.68[a]	6.66 ± 2.50[a]	1.63 ± 1.01[a]	45.67 ± 2.23[a]
Antilopinae (n=17)	38.80 ± 4.78	6.68 ± 1.99	8.86 ± 3.59	1.61 ± 0.88	44.26 ± 4.22
Blesbok (*n=1*)	37.92 ± 4.58[a]	6.47 ± 2.71[a]	8.08 ± 2.71[a]	1.32 ± 0.48[a]	46.19 ± 9.36[a]
Blue wildebeest (*n=2*)	43.38 ± 2.57[a]	7.17 ± 1.57[a]	4.84 ± 0.86[b]	1.02 ± 0.41[a]	43.59 ± 4.03[a]
Impala (*n=1*)	36.30 ± 3.65[a]	8.00 ± 0.92[a]	9.19 ± 3.09[a]	2.29 ± 0.64[b]	46.02 ± 2.17[a]
Springbok (*n=5*)	39.48 ± 3.36[a]	6.59 ± 1.57[a]	9.00 ± 3.24[a]	1.02 ± 0.59[a]	43.47 ± 3.85[a]
Gemsbok (*n=3*)	37.23 ± 6.12[a]	5.46 ± 1.34[a]	11.24 ± 3.00[a]	2.22 ± 0.94[b]	44.86 ± 4.65[a]
Southern lechwe (*n=4*)	34.31 ± 2.98[b]	8.41 ± 1.85[a]	11.78 ± 1.93[a]	2.01 ± 0.59[b]	43.49 ± 3.76[a]
Defassa waterbuck (*n=1*)	43.49 ± 2.29[a]	3.76 ± 0.55[b]	3.89 ± 1.47[b]	2.69 ± 0.71[b]	46.19 ± 2.03[a]
Caprinae (n=2)	37.33 ± 1.02	6.64 ± 0.98	8.29 ± 1.22	1.50 ± 0.40	46.24 ± 2.22
Barbary sheep (*n=1*)	37.01 ± 0.73[a]	5.82 ± 0.55[a]	7.14 ± 0.40[a]	1.65 ± 0.43[a]	48.36 ± 1.09[a]
Mufflon (*n=1*)	37.59 ± 1.23[a]	7.29 ± 0.71[b]	9.20 ± 0.73[b]	1.37 ± 0.37[a]	44.55 ± 0.93[b]

respectively for Bovinae, Caprinae and Antilopinae). Similarly, the estimated average SMI at death was respectively 50.0%, 45.1% and 52.3%.

The comparison of the decay of SMI over time and the estimated average SMI at death, as performed previously, showed no statistically significant difference between the subfamilies tested (Figure 2B).

The decline in SMI was mainly due to the decline in total motility. The SMI at the estimated time of death of the donors was quite low; this was due to the slow speed of the epididymal sperm cells studied. Their speed during the first evaluation was about 2, and declined quickly, most of the sperm (43%) analysed being classified as 1/5 24 hours after the death of donors.

Morphology

To better compare the changes of sperm morphology over time, the results were collated into 24-hour intervals: 0 – <24h, 24 – <48h, 48 – <72h, 72 – <96h, 96 – <120h, 120 – <144h, 144 – <168h, and >168h. The proportions of the different categories of sperm morphology used in this study were not constant over the conservation of epididymes at low temperature (Table 1). Thus, there was a modest reduction in the proportion of sperm with a cytoplasmic droplet; this decrease was statistically significant from 168 hours (seven days) compared with the first values (0–24h). Abnormalities of the head and flagellum and flagellumless spermatozoa increased over time, with a significant difference from 168 hours (seven days).

The proportion of normal spermatozoa collected from epididymes stored at low temperature was independent of time since the estimated time of death of the animal regardless of family or subfamily.

The change in the proportion of different categories of morphology over time for Antilopinae and Caprinae generally followed the same trends as those described above for the Bovidae. The variation among Bovinae appeared earlier; the proportion of sperm with a cytoplasmic droplet decreased significantly from 120 hours (five days) after death. The increase in the proportion of flagellumless sperm was significant at 144 hours (six days).

The different morphology categories used did not appear in the same proportions (Table 2); the most frequently found anomaly was the presence of a cytoplasmic droplet, irrespective of its location, proximal or distal; this was found in 44.8 ± 3.8% of the spermatozoa observed, with a range of 34.0 to 59.5%. This was comparable between the different subfamilies, and no species could be distinguished statistically. The slightly lower average value observed in the mufflon seems more related to a weakness of sampling within Caprinae (one representative for both species).

Morphologically normal spermatozoa represented the second largest category, with an average proportion of 38.5 ± 4.4%, and a range of 28.9 to 48.2%. The species characteristics that emerged from our data included a smaller proportion of normal spermatozoa in lechwes within Antilopinae; however, this may be the consequence of a distortion caused by a delayed first examination for two of the four representatives of this species.
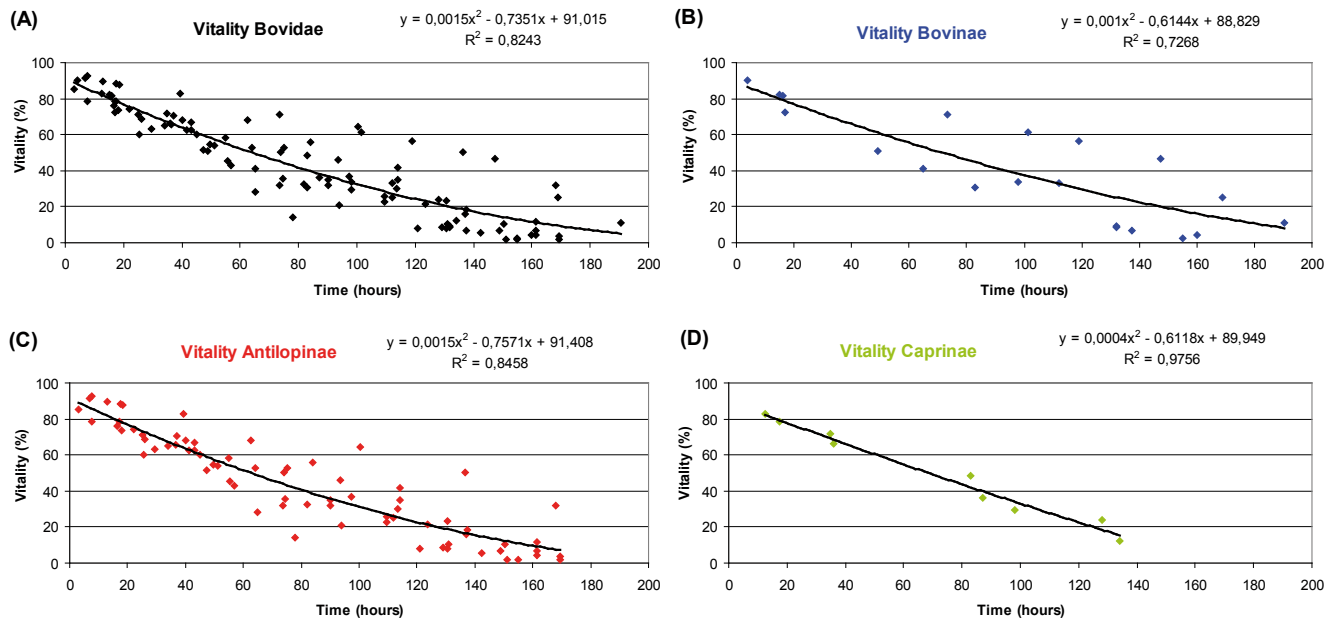
Figure 3. Epididymal sperm vitality over time. (A) Family Bovidae. (B) Subfamily Bovinae. (C) Subfamily Antilopinae. (D) Subfamily Caprinae.

The same observation can be made for the greater kudu, which had a significantly lower proportion of normal spermatozoa within Bovinae.

The other morphology categories studied – head and flagellum abnormalities and flagellumless sperm – were much less frequent. Thus, flagellum abnormalities, among which were recognised bent, coiled or shortened flagella, were found in 8.5% ± 3.4 (range 2.6 to 15.7%). Two species, Cape eland and Defassa waterbuck, showed significantly reduced proportions in comparison with other species of their respective subfamilies; however, these species were each represented in our study by only one individual, and so this result may be due to individual differences. On the other hand, we could also assume the same bias in the differences observed in greater kudu and mufflon.

Head abnormalities (abnormal acrosome, small and narrow heads, enlarged or pear-shaped heads, two-headed spermatozoa) accounted for 6.8 ± 2.0% of the spermatozoa observed, with values ranging from 2.9 to 12.1%. Keeping in mind the same considerations as above, no clear species characteristics emerged. Finally, flagellumless sperm were rarely detected: 1.6 ± 0.8% (range 0 to 4.1%).

Vitality

The percentage of live sperm collected from epididymes stored at low temperature decreased significantly over time (Figure 3.A). The decrease was statistically significant from the fourth day, and a significant decrease was observed after six days.

The decrease in sperm vitality was approximated by a polynomial trend line with a high coefficient of determination (R^2 = 0.824). The estimated percentage of epididymal sperm alive at the time of death of the donors was estimated at 91.0%.

The decrease in epididymal sperm vitality over time was comparable for each subfamily (Figure 3B, C and D). It thus appears that the approximation of this decline follows, as for the Bovidae family level, a polynomial decay with high coefficients of determination (R^2 = 0.727, 0.976 and 0.846 respectively for the Bovinae, Antilopinae and Caprinae). Similarly, the rates of live epididymal sperm estimated at death were respectively 88.83%, 91.41% and 89.95%. The comparison of the decrease in epididymal sperm vitality over time, via the statistical comparison of the trend lines and their standard deviation, showed no statistically significant differences between the subfamilies.

Seasonality

With analysis extending over a full year, this study covered a breeding season (autumn/winter) and a period of no sexual activity (spring/summer). The decrease in total sperm motility and vitality over time showed no statistically significant seasonal effects. Similarly, no significant seasonal difference was observed in the percentages of various morphologies recorded (Table 3).

Thus, it appears that the quality of epididymal sperm (motility, vitality, morphology) was not influenced by season – autumn/winter or spring/summer – in captive exotic Bovidae under the conditions of this study.

Table 3. Morphology of epididymal sperm according to season. Results are expressed as percentages (%), and presented as mean ± standard deviation.

	Morphology				
	Normal	Head abnormalities	Flagellum abnormalities	No flagellum	Cytoplasmic droplet
Spring/summer	38.29 ± 4.18	6.60 ± 1.62	8.78 ± 3.20	1.69 ± 0.80	45.13 ± 4.17
Autumn/winter	38.67 ± 4.71	6.94 ± 2.28	8.27 ± 3.60	1.52 ± 0.83	44.40 ± 3.52

In the collection of species studied here, only greater kudus, springboks, impalas, Barbary sheep and muflons are seasonal breeders. The analysis may have masked seasonal effects, but the numbers of donors from seasonally breeding species was not sufficient for statistical analysis.

Discussion

The conservation of epididymes at 4° C was found to be a satisfactory storage temperature for the sperm of many species of wild ruminants. This has several advantages (Bartels et al. 1999; Bezuidenhout et al. 1999; Bisset et al. 2005 ; Chatiza et al. 2011; Kilian et al. 2000; Martinez et al. 2008; Martinez-Pastor et al. 2005; Soler et al. 2003). First, cold rooms and refrigerators maintain a stable conventional temperature of around 4–6° C, making them appropriate facilities for storage of dead animals or organs over short periods. Secondly, a temperature of about 4° C can be maintained in shipments with suitable devices, and could be used for the transportation of spermatozoa between institutions.

The results of this study show a gradual decrease in epididymal sperm motility and vitality during their storage at 4° C following the death of captive wild Bovidae. The results obtained are comparable to those already published on African wild ruminants suggesting a survival of epididymal sperm three to four days post-mortem when stored at 4° C (Bezuidenhout et al. 1995; Bisset et al. 2005; Friedmann et al. 2000; Kilian et al. 2000; Martinez-Pastor et al. 2005); this decrease appears species-dependent, some studies reporting the absence of sperm motility after three days in gemsboks and roan antelopes (*Hippotragus equinus*) (Kilian et al. 2000). The average epididymal sperm vitality at death is estimated at approximately 91% in this study. This value is consistent with those already published in bovids, between 82 and 99% (Blash et al. 2000; Bissett et al. 2005; Chatiza et al. 2011; Herrick et al. 2004; Lambrechts et al. 1999). This value is slightly lower in Cervidae: 70–79% (Martinez-Pastor et al. 2005; Santiago-Moreno et al. 2006; Soler et al. 2003).

Live sperm could be found more than a week after death (about 10% of sperm were still alive seven days post-mortem), a few days more than the results reported by Bezuidenhout et al. (1995), who found 10% remaining vitality after five days of storage in African buffaloes (*Syncerus caffer*).

The average epididymal sperm motility in this study approached about 74% at the time of death, which is comparable to previously published data in ruminants (between 70 and 86%: Bissett et al. 2005; Chatiza et al. 2011; Martins et al. 2007; Saragusty et al. 2006; Santiago-Moreno et al. 2006). Total motility decreased faster than vitality, and 10% of motile sperm remained after five days in this study. Bissett et al. (2005) found 10% mobile sperm cells remaining after four days in elands.

The Sperm Motility Index has been used in several previous studies on wild ruminants (Herrick et al. 2004; Soler et al. 2003). The SMI at death is estimated at about 60%, with an exception reported for springbok, for which it is around 46% (Herrick et al. 2004). The value obtained in our study is close to 47%. The SMI decreases significantly over time among Cervidae (Soler et al. 2003), and reaches about 44% after four days of storage. On the fourth day, the SMI calculated for the Bovidae in this study approached 10%, which appears significantly lower than the data published by Soler et al. (2005). Lower values derived from our cases could be related to a bias induced by the experimenter's experience, or to an overall decrease in SMI among individuals in captivity; we have not been able to test this hypothesis because, to our knowledge, there is no calculated SMI in captive wild ruminants.

It has previously been reported that sperm collected from the scimitar-horned oryx (*Oryx dammah*) (Roth et al. 1999), gaur (*Bos gaurus*) (Hopkins 1988) and European mufflon (Garde et al. 1995) with a motility of 55–70% and a vitality of 60–75% were able to fertilise heterologous oocytes. By using the same percentages from our results, epididymal sperm collected 24 to 48 hours after death could retain their fertilising capacity. This is consistent with the time of collection recorded by Hopkins et al. (1999) and Garde et al. (1995), performed at 27 and 40 hours post-mortem respectively.

The decline of motility and vitality of spermatozoa collected post-mortem from epididymes stored at low temperature may be influenced by several factors. At 4° C, spermatozoa are metabolically active, but to a lesser extent than when kept at their optimum temperature range (around 35 ° C) (Bisset et al. 2005; Chatiza et al. 2011); nevertheless, they continue their maturation, which requires energy, using resources not renewed following the death of the animal, and this can lead to the death of many gametes.

Other physicochemical factors have also been proposed. In Cervidae, the presence of a protective factor – lecithin – was suggested, its presence being measured during three days post-mortem. The disappearance of this molecule is accompanied by a significant decrease in epididymal sperm motility and vitality (Soler et al. 2003). In Bovidae, the significant decrease in motility and vitality from the third to fourth days could be related to the degradation of this protein in the cauda epididymis; nevertheless, no data could be found on lecithin in this family. Other factors are also suspected; pH and osmolarity in the seminiferous tubules, and their increase over time, appear to be important elements during the storage of sperm and may play a negative role on their quality. The causes of these variations are attributed to aging sperm and tissue breakdown, the degeneration of the seminiferous tubules starting 18 hours after death (Martinez-Pastor et al. 2005). The desiccation of epididymal sperm during their storage could also be an accelerating factor in the decline in gamete quality; to avoid this, many experimenters have placed the epididymes collected in waterproof bags (Bissett et al. 2005; Martinez-Pastor et al. 2005; Soler et al. 2003). We chose to keep the epididymes in their tunica and not to cut the albuginea to reduce desiccation of the underlying tissues.

Species appears to be a determinant of varying sperm quality. In wild Bovidae, Schiewe et al. (1991) have shown a significant difference between the quality of sperm obtained by electro-ejaculation in two African species in situ: a total motility of 91% was found in the blue wildebeest, but 59% in greater kudu; similarly, the proportion of normal spermatozoa was respectively 96% and 88%. Significant interspecific differences have also been reported between blesbok, Cape buffalo, springbok and blue wildebeest (Herrick et al. 2004) and between red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*) (Martinez-Pastor et al. 2005).

At individual and at population levels, many external factors can influence sperm quality, including male provenance – differences have been observed between individuals of the same species but from different populations (Brown et al. 1991; Martinez-Pastor 2005; Soler et al. 2003). Sperm quality is also influenced by social status; several studies have shown significant selective suppression of the hypothalamic–pituitary axis inducing a reduction in sperm quality in subordinate male wild ruminants (Bisset et al. 2005; Brown et al. 1991). The precise social status of the animals in our study is unknown and may be an important bias in the analysis of sperm quality. On the other hand, environmental conditions, and in particular food and water quality and availability, and temperature, are significant sources of variation (Chatiza et al. 2011).

Several publications have reported an increase in morphological abnormalities in sperm collected from epididymes stored at low temperature (Bartels et al. 1999; Bissett et al. 2005; Martinez-

Pastor et al. 2005). In Cervidae, it appears that the increase in morphological abnormalities is significant from the fourth day (Martinez-Pastor et al. 2005). The detection of a cytoplasmic droplet (proximal or distal) is reported as the most frequent morphology in epididymal sperm (Herrick et al. 2004; Martins et al. 2007; Martinez et al. 2008). The morphology distribution published for the mountain gazelle (*Gazella gazella*) and dorcas gazelle (*Gazella dorcas*) (Saragusty et al. 2006) approaches our results except for spermatozoa with a cytoplasmic droplet (in this study, between 3.5 and 17%). This difference could be caused by a divergent definition of normal spermatozoa: these authors might have considered sperm with distal cytoplasmic droplets as normal. Bissett et al. (2005) compared the changes in proximal and distal cytoplasmic droplets over time; a significant reduction in sperm with distal cytoplasmic droplets was detected, while the proportion of sperm with a proximal droplet remained stable. As indicated in our results, head and flagellum abnormalities are reported as rarely present and increase moderately over time (Bernard et al. 2005; Martinez-Pastor et al. 2005).

The influence of season on sperm quality has been studied in Cervidae; motility and sperm without morphological abnormalities are at a maximum during the breeding season (Coloma et al. 2011; Goeritz et al. 2003, Martinez-Pastor et al. 2005). Ghosh and Fischer (1989) found that most samples collected from fallow deer (*Dama dama*) by electro-ejaculation in June–July are azoospermic. In wild Bovidae, knowledge is more limited and sometimes contradictory between publications. The diameter of the seminiferous tubules or testes, sperm count per ejaculate and sperm motility appear lower outside the breeding season in the springbok, blesbok, impala, greater kudu, blue wildebeest, hartebeest (*Alcelaphus caama*) and the African buffalo (Skinner 1971; Skinner et al. 1973). On the other hand, nyalas (*Tragelaphus angasi*) show no annual reproductive cycle, but a bimodal pattern with two breeding peaks in spring and autumn, the sperm parameters following this bimodal distribution respectively; the authors argue that this physiological model could be valid for Tragelaphini, including greater kudu and sitatunga (Anderson 1979). The lack of seasonality in sperm parameters observed in our study may have several explanations. Firstly, the relatively large number of species included compared to the number of individuals may introduce bias with reference to the physiological differences mentioned above. The number of representatives per species being insufficient, we have not been able to identify species characteristics to compare with data in the literature. The reports described above are all in situ studies, and the effects of captivity (chronic stress, dietary needs, social structure and altered photoperiod) experienced by the subjects in this study could affect sperm quality.

All manipulations and microscopic examinations were conducted by a single experimenter, eliminating interindividual variability. Nevertheless, it would have been interesting to establish a comparison with a computerised technique, thereby detecting a possible margin of error. It would also be useful to better compare these results to previously published data.

Estimating the time of death for 66.7% of the animals in our study produced a significant bias in the further processing of data over time; however, the data are consistent with previously standardised published data, which suggests that this approximation is acceptable.

Cryopreservation of epididymal spermatozoa collected post-mortem has been reported in several species: ram (Blash et al. 2000), bull (Martins et al. 2007), springbok, impala, blesbok (Chatiza et al. 2011) , Iberian ibex (*Capra pyreneica*) (Coloma et al. 2011, Santiago-Moreno et al. 2006), mountain and dorcas gazelles (Saragusty et al. 2006), Cape buffalo (Lambrechts et al. 1999), blesbok, springbok, Cape buffalo, blue wildebeest, gemsbok and Mhorr gazelle (*Gazella dama mhorr*) (Chatiza et al. 2011).

Cryopreservation of epididymal sperm results in a decrease in motility and vitality and causes damage to the acrosome membrane (Chatiza et al. 2011; Coloma et al. 2011; Herrick et al. 2004; Martins et al. 2007; Saragusty et al. 2006; Santiago-Moreno et al. 2006). However, several publications describe the conservation of fertilising capacity of epididymal sperm after freezing–thawing. Cryopreservation of spermatozoa outside the breeding season has given good results in several wild Bovidae species (Saragusty et al. 2006). Finally, Chatiza et al. (2011) showed that the media used for in vitro fertilisation techniques in cattle can be used in some species of wild Bovidae, although improvements are needed. Despite all this lack of specific knowledge, fertilisation has been reported after post-mortem epididymal sperm collection, cryopreservation and artificial insemination: heterologous fertilisation with gaur sperm (Hopkins et al. 1988) and homologous with bull sperm (Martins et al. 2007), and recorded births of mufflons (Blash et al. 2000) and a Cape eland (Bartels et al. 2001).

This study describes the changes in epididymal sperm quality at low temperature in species for which no data has yet been published: Southern lechwe, Defassa waterbuck, sitatunga and Barbary sheep. This work also represents the first evaluation of post-mortem epididymal sperm collection by aspiration and storage at low temperature in captive wild Bovidae. This technique can be easily done in zoos with little equipment and without precise knowledge of the time of death. These observations could lead many institutions to perform gamete collection on their dead male bovids, and thus, ultimately, develop assisted reproductive techniques from epididymal sperm and increase the genetic material available in zoos. Nevertheless, the increasing use of these techniques demands more work on the reproduction physiology of many wild species. Similarly, adapting cryopreservation techniques for each species is necessary. However, it would be worthwhile to extend this study by performing fertility tests over time (via heterologous fertilisation, for example) because it has been observed in previous studies that fertilising capacity decreases quicker than motility or vitality during sperm storage.

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